REMARKS

This amendment responds to the Office Action mailed December 19, 2002. In the Office Action the Examiner:

- rejected claims 17-25 as being indefinite under 35 U.S.C. 112, second paragraph, and

- rejected claim 17-25 as not being enabled under 35 U.S.C. 112, first paragraph, and

- rejected claims 17-25 under 35 U.S.C. 102(b) as anticipated by Murphy *et. al.* (WO 98/53078).

- After entry of this amendment, the pending claims are: claims 17-26.

Applicants have amended claims 17-25 and added new claim 26. In particular, the amended claims relate to a recombinant parainfluenza virus encoding one or more heterologous sequences that have been added to said virus genome or substituted for nucleotide sequences of said virus genome. Support for the claims as amended is provided, *inter alia*, in the specification as described in Table 1 below. Accordingly, no new matter has been added.

TABLE 1	
CLAIM	**SUPPORT IN THE SPECIFICATION**
17	Page 13, lines 21-24; Page 10, lines 15-21; Page 14, lines 1-5 & 25-29
18-20, 24	Page 7, lines 1 & 2 Page 7, lines 11-16 Page 27, lines 3-7 Page 8, lines 24-28
21	Page 15, lines 1-3
22	Page 13, lines 24-29
25 - 26	Page 30, lines 8-13

THE REJECTIONS UNDER 35 U.S.C. § 112, SECOND PARAGRAPH, SHOULD BE WITHDRAWN

Claims 17-25 are rejected under 35 U.S.C. § 112, second paragraph, as being allegedly indefinite. The claims have been amended to more particularly point out and distinctly claim the invention. In particular, the claims have been amended to reflect that the claimed invention relates to recombinant parainfluenza virus that expresses one or more heterologous sequences.

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Contrary to the Examiner's contention, Applicants respectfully point out that the specification clearly describes recombinant parainfluenza viruses that have been engineered to express heterologous sequences (see, *e.g.*, page 7, lines 1 & 2; page 7, lines 11-16; page 27, lines 3-7; and page 8, lines 24-28). The heterologous sequences intended to be expressed by the recombinant parainfluenza viruses of the invention are clearly described by the specification as including those sequences that encode antigens characteristic of a pathogen, such as bacteria and viruses, antigens that are characteristic of autoimmune disease, antigens that are characteristic of an allergan, and antigens that are characteristic of a tumor (see *e.g.*, page 14, lines 26-30 in the specification). In particular, the specification describes the expression of heterologous sequences, such as gene sequences from other strains of parainfluenza or sequences derived from other viruses. Moreover, the specification provides working examples that demonstrate a recombinant parainfluenza virus engineered to express a heterologous sequence or gene, *i.e.*, a parainfluenza virus engineered to express a sequence derived from another species of parainfluenza virus, namely bovine parainfluenza virus engineered to express heterologous sequences derived from human PIV encoding the F and HN genes.

As described in the specification, the presence of the heterologous sequences in the recombinant parainfluenza virus was confirmed by DNA sequence analysis (see, *e.g.*, page 31, lines 9-13 of the specification). Expression of the heterologous sequences was confirmed by the generation of a strong immune response specific to the heterologous sequences by the recombinant parainfluenza virus once the recombinant virus was administered to a host organism. Applicants direct the Examiner's attention to Table 2 on page 34 of the present application. As shown in Table 2 and as discussed in the specification on lines 17-18 of page 34, the bPIV3/hPIV3 virus generates a strong antibody response against human PIV3 -- polypeptides, demonstrating that the human PIV3 sequences of the bPIV3/hPIV3 virus, *i.e.*, the heterologous sequences, are indeed expressed. Without the expression of the heterologous sequences, *i.e.*, the human PIV3 sequences, the hamsters used in this assay would not have expressed antibodies against human PIV3. Furthermore, Applicants respectfully point out that viruses lacking one or more of the PIV genes would not be able to undergo successive rounds of replication (see, *e.g.*, in the specification at page 28, line 32 and continuing on page 29, lines 1 & 2). One skilled in the art would be able to easily understand the claimed invention when reading the claims in light of the specification. Therefore, Applicants respectfully contend that the specification clearly describes and exemplifies the invention as claimed.

The Examiner contends that claim 22 is indefinite for recitation of "mutations or modifications" and "enhanced antigenicity," stating that these terms are relative and subject to varied interpretation. Applicants respectfully disagree. The test for definiteness is

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whether those skilled in the art would be apprised of the scope of what is claimed (see M.P.E.P. 2173.02 citing *Solomon v. Kimberly-Clark Corp.*, 216 F.3d 1372, 1379, 55 USPQ2d 1279, 1283 (Fed. Cir. 2000); *In re Larsen*, No. 01-1092 (Fed. Cir. May 9, 2001) (unpublished)). Applicants assert that the claims as written would be clear to the skilled artisan, especially when taken in light of the specification and the claimed technology. The specification, coupled with the knowledge in the art as of the effective filing date of the instant application provides one of skill in the art with ample guidance to determine the metes and bounds of the claims. The claims clearly set forth what the Applicants regard as their invention. Applicants also believe that the scope of the subject matter embraced by the claims is clear. Moreover, one of skill in the art, coupled with the teaching in the specification, would be able to understand the claimed invention.

The terms "mutation" and "modification" both have unambiguous meanings to one practicing routine recombinant DNA techniques and the technology of the invention. Moreover, the term "enhanced antigenicity" is clear when taken in light of the claimed utility of the immunogenic compounds and vaccines of the invention. Applicants respectfully point out that one skilled in the art would know that the enhanced antigenicity of the mutated or modified recombinant virus would be determined relative to the wild type or unmutated or unmodified virus. In response to the Examiner's inquiry about how one might determine "enhancement," Applicants respectfully point out that one skilled in the art would be familiar with methods to determine the successful enhancement of a host response to the recombinant virus of the invention. Moreover, Applicants invite the Examiner's attention to page 34, lines 14-30, and also in Table 2 of the specification, describing the quantitation of serum titers in response to the recombinant virus of the invention.

The Examiner has also rejected claim 23 as lacking antecedent basis. In response, Applicants have amended claim 23 to properly place it in a condition for allowance.

For the forgoing reasons, Applicants respectfully request that the Examiner's rejection of claims 17-25 under 35 U.S.C. § 112, second paragraph be withdrawn.

THE REJECTIONS UNDER 35 U.S.C. § 112, FIRST PARAGRAPH, SHOULD BE WITHDRAWN

Claims 17-25 are rejected under 35 U.S.C. §112, first paragraph, for alleged failure of the specification to provide enablement for the full scope of the claims. The Examiner contends that the specification fails to provide guidance to enable one skilled in the art to practice the claimed invention. The invention as claimed is fully enabled by the instant specification and as such, the rejection under 35 U.S.C. § 112, first paragraph, should be withdrawn.

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The claimed invention relates to recombinant parainfluenza virus that expresses one or more heterologous sequences. Contrary to the Examiner's position, the instant application provides all the information required by one of skill in the art to construct and generate a recombinant virus with one or more heterologous sequences. Applicants respectfully assert that the specification, coupled with the state of the art as of the effective filing date of the instant application, fully enables one of skill in the art to make, use, and practice the invention as claimed without undue experimentation.

The specification, as originally filed, describes the claimed invention as relating to a recombinant parainfluenza virus that expresses one or more heterologous sequences. According to the specification, the recombinant parainfluenza viruses have utility as immunogenic compounds and as vaccines. For example, the specification clearly teaches that the recombinant virus of the invention can be used to produce immunogenic compounds to elicit an antibody response (see, *e.g.*, page 1, lines 18-20; page 20, lines 18-20; and also on page 27, lines 12-15 of the specification). The specification also teaches the significance of preserving the immunogenicity of the recombinant virus in order to enhance its utility (see, e.g, page 29, lines 15-19). Applicants assert that the specification clearly describes the viruses of the invention as immunogenic compounds and as vaccines.

Applicants point out that the specification clearly describes how to make the claimed invention. A specification that discloses at least one method for making and using the claimed invention enables the entire scope of the claims and satisfies the enablement requirement of 35 U.S.C. 112 (see MPEP 2164.01(b) citing *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18,24 (CCPA 1970)). Applicants respectfully assert that the specification, as originally filed, meets this standard. For example, the specification describes methods for the insertion of a heterologous sequence into the nonsegmented genome of PIV (see, *e.g.*, page 19, in section 5.1.1 of the specification). Furthermore, the specification describes methods for the insertion of the heterologous sequence into the coding sequences of the viral genome, such as those encoding the HN gene (see, *e.g.*, page 20, in section 5.1.2 of the specification). Moreover, the specification clearly describes that the resultant virus would be immunogenic if the heterologous sequence is expressed on the outside surface (see section 5.1.2 of the specification). The specification also provides working examples that exemplify the claimed invention (see, *e.g.*, Example 1 on page 30 of the specification). In brief, the example clearly sets out the method of construction of a recombinant parainfluenza virus that expresses heterologous sequences. The specification also describes the construction of a recombinant bovine parainfluenza virus that expresses heterologous sequences from another strain of parainfluenza, *e.g.*, human PIV F and HN genes.

Applicants point out that the specification clearly describes how to use the claimed invention. If a statement of utility in the specification contains within it a

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connotation of how to use, and/or the art recognizes that standard modes of administration are known and contemplated, 35 U.S.C. 112 is satisfied. *In re Johnson*, 282 F.2d 370, 373, 127 USPQ 216, 219 (CCPA 1960); *In re Hitchings*, 342 F.2d 80, 87, 144 USPQ 637, 643 (CCPA 1965). See also *In re Brana*, 51 F.2d 1560, 1566, 34 USPQ2d 1437, 1441 (Fed. Cir. 1993). Applicants respectfully assert that the present specification clearly teaches how to use the recombinant virus of the invention as immunogenic compounds as well as vaccines. Moreover, in light of the teaching in the specification, one skilled in the art would be able to use the invention absent undue experimentation. The specification clearly teaches that recombinant viruses of the invention can be used as immunogenic compounds in a host in order to elicit an immune response (see Example 4, on page 32). The present specification describes the strong protective response that was elicited in an animal model against the heterologous sequences expressed by the recombinant parainfluenza viruses of the invention. Moreover, the data in Table 2, on page 34 of the specification, demonstrate that the antibody response generated in hamsters by the recombinant virus is "as strong an antibody response against hPIV3" as that generated by wild type hPIV3 in the same host (see also, page 33, lines 27-30). For example, Table 2 shows the antibody response generated in the hamsters upon being infected with the different PIV3 strains. As is indicated by the data in Table 2, the serum titers for hamsters infected with the recombinant bPIV3/hPIV3 virus was equivalent to those infected with wild type hPIV3.

Further, the Examiner contends that "Applicants own data indicates induction of antibodies, but that this is not complete protection." The Examiner also contends that, "There are no challenge studies present, even for parainfluenza." As provided in the specification, Applicants have demonstrated that the recombinant virus of the invention is immunogenic and also generates a strong antibody response (see, *e.g.*, Example 4, on page 32 of the specification, with specific references also cited herein). Applicants point out that no undue experimentation is required to determine whether the claimed recombinant parainfluenza viruses are immunogenic, *e.g.*, whether they cause the generation of an antibody response in a host. Applicants assert that it is an accepted view in the art that the generation of specific antibodies in response to administering an immunogenic compound correlates with the generation of immunity against the pathogen. Applicants also point out that "undue experimentation is experimentation that would require a level of ingenuity beyond that to be expected of one of ordinary skill in the art." *Fields v. Conover*, 443 F.2d 1386, 1390-1391, 170 USPQ 276, 279 (CCPA 1971). It would be considered routine experimentation in order to demonstrate that the antibody response generated by a host upon infection with the recombinant virus of the invention could confer immunity. The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *In re Certain Limited-Charge Cell Culture Microcarriers*,

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221 USPQ 1165, 1174 (Intl Trade Comm 1983), *aff'd sub nom., Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985). See also *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. Applicants assert that one skilled in the art would know how to formulate vaccines and to conduct suitable assays without undue experimentation.

Furthermore, Applicants point out that the art also demonstrates that the claimed compounds are immunogenic. The specification, as originally filed, does fully enable the skilled artisan to make and use viruses corresponding to the scope of the presently pending claims. In support, Applicants submit a copy of Haller *et al.*, 2000, J. Virology 74:11626-11635 **(attached as Exhibit B; "Haller *et al.*")**. Haller *et al.* show that a virus of the present invention can be successfully used to protect an animal model from viral infections. Haller *et al.* describes the use of a recombinant bovine/human PIV3 to protect hamsters from challenge with hPIV3. The recombinant virus in Haller *et al.* was constructed in accordance with the guidance provided in the instant application. As discussed on page 11632, right column to page 11633, left column, and as shown in Table 4 of Haller *et al.* the bovine/human PIV3 protects hamsters from challenge with human PIV3. In particular, Applicants invite the Examiner's attention to the last paragraph of the right column at page 11632: "All of the immunizing viruses (bPIV3, r-PIV3, hPIV3, and bovine/human PIV3) provided complete protection from challenge with wt hPIV3 (Table 4)." Clearly, a recombinant virus that has been constructed according to the disclosure of the instant specification provides complete protection from viral infection. Thus, the instant specification is enabling for the claimed invention.

The Examiner incorrectly contends that Applicants have not taught expression of any heterologous gene. In fact, as discussed above, Applicants have not only taught but have also exemplified the expression of heterologous sequences.

For the forgoing reasons, Applicants respectfully request that the rejections under 35 U.S.C. § 112, first and second paragraphs, of claims 17-25 be withdrawn.

THE REJECTIONS UNDER 35 U.S.C. § 102 SHOULD BE WITHDRAWN

The Examiner has rejected claims 17-25, which are drawn to chimeric parainfluenza viruses as anticipated by Murphy et al (WO 98/53078; "Murphy"). In particular, the Examiner points out that Murphy teaches "[...] that a bovine parainfluenza virus may be modified to comprise heterologous genes including glycoproteins that can be substituted from human PIVs that would induce an immunogenic response." Furthermore, the Examiner contends that the above cited art does not exclude the Kansas-strain and therefore, Murphy *et. al.* are entitled to the broadest possible interpretation, including that of the Kansas-strain.

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Applicants submit that the Murphy reference does not anticipate their claims. Applicants respectfully point out that a claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). Furthermore, "the identical invention must be shown in as complete detail as is contained in the … claim." *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236 (Fed. Cir. 1989). Applicants point out that Murphy does not describe the use of the Kansas-strain and therefore does not anticipate the claims of the present invention.

Moreover, the Examiner's contention that the Murphy reference is anticipatory because it does not "say they want to be excluded of Kansas-strain or be limited to only one strain," is erroneous. While M.P.E.P. 2131.02 states that "a genus does not always anticipate a claim," that is not to say that the lack of exclusion of a species makes the species anticipated by the genus. To the contrary, Applicants respectfully point out that a reference is anticipatory if it clearly names the claimed species. *In re Sivaramakrishnan*, 673 F.2d 1383, 213 USPQ 441 (*Sivaramakrishnan*). See also MPEP § 2131.02. In *Sivaramakrishnan*, the sole reference relied upon by the Examiner disclosed a large number of compounds under a generic formula in addition to a particular species of the genus. The court in *Sivaramakrishnan* found that the § 102 rejection was proper only because the species was "specifically" named in the prior art reference. In the instant case, the claimed species is not specifically named in Murphy, and as such, the 102 rejection in view of Murphy is not proper. Thus, Applicants respectfully request that the rejection under 35 U.S.C. § 102 be withdrawn.

THE OBJECTION UNDER 37 C.F.R. 1.75(c) SHOULD BE WITHDRAWN

The Examiner has objected to claims 24 and 25 under 37 C.F.R. 1.75(c) as being of improper dependent form for failing to further limit the subject matter of a previous claim. Applicants respectfully disagree with the Examiner's objection. Claim 24 clearly limits claims 17-23 from which it depends by specifying that the heterologous sequences substitute both the F and the HN genes of the genome of bovine PIV3. Claim 25 clearly limits claims 17-23 from which it depends by specifying the nucleotide positions of the genome of PIV3 where the heterologous sequences are inserted.

The Examiner's contention that there is no antecedent basis for "heterologous sequence" in claim 20 is also erroneous. Applicants respectfully point out that claim 20 ultimately depends on claim 17 which cites "heterologous sequences." Therefore, there is proper antecedent basis for claims 24 and 25 to depend from claim 20.

For the forgoing reasons, Applicants respectfully request that the objection to claims 24 and 25 under 37 C.F.R. 1.75(c) be withdrawn.

CONCLUSION

Applicants respectfully request that the amendments and remarks of the present response be entered and made of record in the instant application. Withdrawal of the Examiner's rejections and an allowance of the application are earnestly requested. If any issues remain in connection herewith, the Examiner is respectfully invited to telephone the undersigned at (212) 790-9090, if a telephone call could help resolve any remaining items.

Respectfully submitted, by: *Jacqueline Benn*
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Date: June 19, 2003

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17.   A recombinant parainfluenza virus comprising:

(i) nucleotide sequences of a Kansas-strain bovine parainfluenza virus type 3 genome; and

(ii) one or more heterologous sequences, wherein said one or more heterologous sequences have been added to said virus genome or have been substituted for nucleotide sequences of said virus genome.

18.   The recombinant parainfluenza virus of claim 17, wherein the heterologous sequences are that of a human parainfluenza virus.

19.   The recombinant parainfluenza virus of claim 18, wherein the heterologous sequences encode the F and HN glycoproteins of a human parainfluenza virus.

20.   The recombinant parainfluenza virus of claim 19, wherein the F and HN glycoproteins of an hPIV are that of a human parainfluenza virus type 3.

21.   The recombinant parainfluenza virus of claim 17, wherein the heterologous sequences are that of an influenza virus or of a respiratory syncytial virus.

22.   The recombinant parainfluenza virus of claim 17, wherein the Kansas-strain bPIV3 backbone contains mutations or modifications, in addition to heterologous sequences, which result in a recombinant virus having a phenotype more suitable for use in vaccine formulations such as an attenuated phenotype or a phenotype with enhanced antigenicity.

23.   A recombinant parainfluenza virus comprising:
(i) the genome of Kansas-strain bovine parainfluenza virus type 3; and
(ii) one or more heterologous sequences, wherein said one or more heterologous sequences have been added to said genome.

24.   The recombinant parainfluenza virus of any one of claims 17-23, wherein said heterologous sequence substitutes both the F and the HN gene of Kansas-strain bovine parainfluenza virus type 3.

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25.   The recombinant parainfluenza virus of any one of claims 17-23, wherein said heterologous sequence is added at a nucleotide position of Kansas-strain bovine parainfluenza virus type 3 selected from the group consisting of nucleotide position 5041, the HN gene, and nucleotide position 8529.

26.   A recombinant parainfluenza virus comprising

(i)   nucleotide sequences of Kansas-strain bovine parainfluenza virus type 3 genome comprising 1-5041 and 8529-15,456 of the genome of Kansas strain bovine parainfluenza virus type 3; and

(ii)   F and HN gene sequences of human parainfluenza virus type 3.

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Expression of the Surface Glycoproteins of Human Parainfluenza Virus Type 3 by Bovine Parainfluenza Virus Type 3, a Novel Attenuated Virus Vaccine Vector

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Bovine parainfluenza virus type 3 (bPIV3) is being evaluated as an intranasal vaccine for protection against human PIV3 (hPIV3). In young infants, the bPIV3 vaccine appears to be infectious, attenuated, immunogenic, and genetically stable, which are desirable characteristics for an RNA virus vector. To test the potential of the bPIV3 vaccine strain as a vector, an infectious DNA clone of bPIV3 was assembled and recombinant bPIV3 (r-bPIV3) was rescued. r-bPIV3 displayed a temperature-sensitive phenotype for growth in tissue culture at 39°C and was attenuated in the lungs of Syrian golden hamsters. In order to test whether r-bPIV3 could serve as a vector, the fusion and hemagglutinin-neuraminidase genes of bPIV3 were replaced with those of hPIV3. The resulting bovine/human PIV3 was temperature sensitive for growth in Vero cells at 37°C. The replication of bovine/human PIV3 was also restricted in the lungs of hamsters, albeit not as severely as was observed for r-bPIV3. Despite the attenuation phenotypes observed for r-bPIV3 and bovine/human PIV3, both of these viruses protected hamsters completely upon challenge with hPIV3. In summary, bPIV3 was shown to function as a virus vector that may be especially suitable for vaccination of infants and children against PIV3 and other viruses.

In recent years, advances in reverse genetics technology have enabled a number of negative-strand RNA viruses to be genetically altered so that they can function as vectors expressing foreign antigens (23, 26). Nonretrovirus RNA virus vectors have a number of theoretical advantages over DNA virus vectors. (i) This group of viruses does not have a DNA phase in their life cycle, which reduces safety concerns related to integration into human DNA. (ii) Homologous recombination has never been observed among negative-strand RNA viruses, which further reduces safety concerns related to genetic stability. (iii) The insertion of foreign genes into the viral genome would be expected to attenuate the vector, adding an additional safety margin to a vectored negative-strand RNA vaccine. (iv) Negative-strand RNA viruses exhibit a transcriptional gradient from the 3' to the 5' end of the genome that results in a gradual decrease of viral protein levels (6). The level of expression of the introduced foreign gene can therefore be modulated, depending on its position in the genome. In many cases, the foreign proteins, in particular membrane proteins, are incorporated as an integral part of the virion (14, 27). Expression of foreign proteins and/or antigens has now been shown for genetically engineered influenza viruses (22, 30), rabies virus (21), vesicular stomatitis virus (14, 27), measles virus (28), bovine and human respiratory syncytial virus (4, 13), human parainfluenza virus type 3 (hPIV3) (2, 32), simian virus type 5 (11), and Sendai virus (10).

In this study, we report the application of bovine PIV3 (bPIV3) as a virus vector. bPIV3 is an enveloped, negative-strand RNA virus within the family *Paramyxoviridae*. The viral RNA genome of bPIV3 is 15,456 nucleotides (nt) in length and encodes nine structural and nonstructural viral proteins (1)

(Fig. 1A). The linear order of genes in the bPIV3 and hPIV3 genomes is very similar (1, 6) (Fig. 1B). The gene sequences of both bPIV3 and hPIV3 are flanked by two short untranslated regions, a 3' leader and a 5' trailer sequence that harbor the viral replication and transcription signals. Each gene of bPIV3 or hPIV3 is bracketed by conserved gene start and gene stop signals directing initiation as well as termination of virus transcription and polyadenylation of viral mRNAs (12). Viral transcription and replication of both PIV3s involve a multiprotein complex that consists of the viral polymerase (L), the nucleocapsid protein (NP), and the phosphoprotein (P) (1, 18). The matrix (M) protein of PIV3 plays an undefined role in virion assembly and packaging of the viral RNA. In addition, bPIV3 encodes three small viral proteins (C, D, V) of unknown function. Each is derived from the P mRNA by alternative molecular mechanisms (1). hPIV3 also encodes a D protein from the P mRNA by RNA editing, but it appears unlikely that the V open reading frame of hPIV3 is translated, since multiple inframe stop codons would result in the premature termination of protein synthesis (8). The bPIV3 and hPIV3 surface glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins, represent the major viral antigens responsible for eliciting neutralizing antibodies in the host (6). The F and HN proteins of bPIV3 and hPIV3 are 77 and 80% identical at the amino acid level, respectively (33). The HN protein mediates virus attachment to the cell surface as well as virus release, while the F protein is responsible for promoting fusion of infected cells leading to syncytium formation in vitro (6).

The bPIV3 strain (Kansas/15626/84) was originally isolated from a calf in Kansas and is currently being tested in clinical trials as a vaccine to protect young children from disease caused by hPIV3. The rationale for this approach is that bPIV3 is antigenically related to hPIV3 and has been shown to protect nonhuman higher primates and other animals against hPIV3 infection in challenge studies (33). Infection with hPIV3 can result in significant morbidity in children under 3 years of age and is responsible for approximately 20% of hospitalizations

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Expression of the Surface Glycoproteins of Human Parainfluenza Virus Type 3 by Bovine Parainfluenza Virus Type 3, a Novel Attenuated Virus Vaccine Vector

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(Fig. 1A). The linear order of genes in the bPIV3 and hPIV3 genomes is very similar (1, 6) (Fig. 1B). The gene sequences of both bPIV3 and hPIV3 are flanked by two short untranslated regions, a 3' leader and a 5' trailer sequence that harbor the viral replication and transcription signals. Each gene of bPIV3 or hPIV3 is bracketed by conserved gene start and gene stop signals directing initiation as well as termination of virus transcription and polyadenylation of viral mRNAs (12). Viral transcription and replication of both PIV3s involve a multiprotein complex that consists of the viral polymerase (L), the nucleocapsid protein (NP), and the phosphoprotein (P) (1, 18). The matrix (M) protein of PIV3 plays an undefined role in virion assembly and packaging of the viral RNA. In addition, bPIV3 encodes three small viral proteins (C, D, V) of unknown function. Each is derived from the P mRNA by alternative molecular mechanisms (1). hPIV3 also encodes a D protein from the P mRNA by RNA editing, but it appears unlikely that the V open reading frame of hPIV3 is translated, since multiple in-frame stop codons would result in the premature termination of protein synthesis (8). The bPIV3 and hPIV3 surface glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins, represent the major viral antigens responsible for eliciting neutralizing antibodies in the host (6). The F and HN proteins of bPIV3 and hPIV3 are 77 and 80% identical at the amino acid level, respectively (33). The HN protein mediates virus attachment to the cell surface as well as virus release, while the F protein is responsible for promoting fusion of infected cells leading to syncytium formation in vitro (6).

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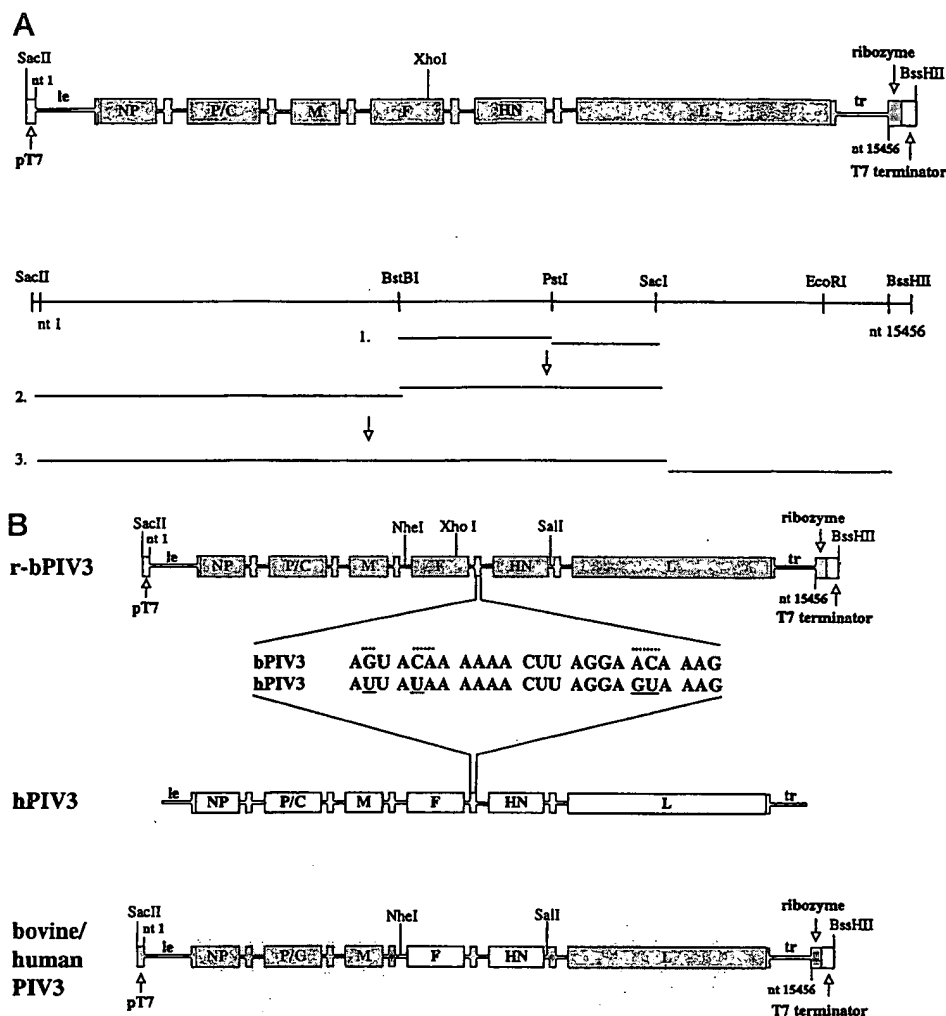† Present address: Mayo Clinic, Rochester, Minn.

FIG. 1. (A) Schematic representation of the r-bPIV3 RNA genome and gene organization. The untranslated 3' leader (le) and 5' trailer (tr) regions flanking the coding sequences are shown. The gene start and gene stop sequences bracketing each viral gene are indicated by black rectangles. The T7 RNA polymerase promoter that precedes nt 1 of the viral genome is shown, as well as the hepatitis delta RZ sequences and the T7 RNA polymerase terminator sequences following nt 15456 of r-bPIV3. The restriction enzyme sites SacII and BssHII, which flank the r-bPIV3 genome, are indicated as shown. The introduced XhoI recognition site located in the F gene of r-bPIV3 is denoted. In addition, the three-step cloning scheme used to obtain the infectious full-length cDNA of r-bPIV3 is shown diagramatically. The major restriction enzyme sites employed to assemble the full-length DNA of bPIV3 are listed. The complete cloning process is described in detail in Materials and Methods. (B) A diagram showing the RNA genomes of r-bPIV3, hPIV3, and bovine/human PIV3. The gene stop and gene start sequences of the F/HN intergenic regions of bPIV3 and hPIV3 are compared. The underlined nucleotides indicate the residues that differ between hPIV3 and bPIV3. The nucleotides overlined with a dotted line are the variable residues in the gene start and gene stop sequences. All of the other nucleotides are conserved between hPIV3 and bPIV3. The restriction enzyme sites NheI in the M/F intergenic region and SalI in the HN/L intergenic region are shown. These restriction enzyme sites were used to delete the bPIV3 F and HN genes and replace them with those of hPIV3.

among young infants and children with respiratory tract infections (7). hPIV3 is a causative agent of bronchiolitis, croup, and pneumonia in children during the first month of life (34). Children are a major factor in introducing hPIV3 infection into the family setting. In the United States, at least 60% of children are infected with hPIV3 by the time that they reach 2 years of age and 80% by 4 years of age (24). hPIV3 frequently recurs in children, although subsequent infections are less severe (5, 9). At present, no vaccine is available to protect children or adults at high risk from respiratory disease caused by PIVs, and no drugs are available for the treatment of disease caused by PIV.

The bPIV3 Kansas/15626/84 strain has been studied as a vaccine candidate against hPIV3 in adults, children, and infants. Results of phase 1 and phase 2 clinical trials in young children between 2 and 60 months of age vaccinated with bPIV3 showed that bPIV3 was infectious, genetically stable, and did not cause lower respiratory disease in children (15, 16). This observation has been attributed to the host cell-restricted disease phenotype observed for bPIV3 in humans. These data suggest that bPIV3 could be an effective and safe vector for delivery of antigens of the human PIV serotypes which cause disease in children.

The initial goal of this study was to demonstrate that bPIV3 can function as a virus vector. The 15,456-nt-long bPIV3 genome was assembled into an infectious cDNA clone from reverse transcriptase PCR (RT-PCR)-derived fragments, and recombinant bPIV3 (r-bPIV3) was rescued by reverse genetics. Sequence comparison of the r-bPIV3 and bPIV3 genomes showed four nucleotide changes present in the r-bPIV3 se-

quence. r-bPIV3 was then analyzed in vitro and in vivo to evaluate the effect of these nucleotide alterations on virus replication. Interestingly, r-bPIV3 displayed a temperature-sensitive phenotype in vitro and was attenuated in small animals. To test the potential of r-bPIV3 as a virus vaccine vector, a chimeric bovine/human PIV3 was constructed by replacing the F and HN genes of bPIV3 with those of hPIV3. The chimeric bovine/human PIV3 also displayed a temperature-sensitive phenotype and was attenuated in small animals, albeit not as severely as r-bPIV3. Both the r-bPIV3 and the bovine/human PIV3 protected small animals from infection with wild-type (wt) hPIV3 in challenge studies. These data support the use of r-bPIV3 as a vector to express and deliver foreign antigens. The application of r-bPIV3 provides an alternative approach to generate novel, live, attenuated vaccines for the protection of children and adults from disease-causing agents.

MATERIALS AND METHODS

Viruses and cells. The bPIV3 (Kansas/15626/84), r-bPIV3, hPIV3 (Washington/47885/57 and Texas/12084/1983), and bovine/human PIV3 were grown in Vero cells in Opti-MEM (Gibco/BRL). The modified vaccinia virus Ankara (MVA-T7), which expresses the phage T7 RNA polymerase (35), was grown in chicken embryonic kidney cells (SPAFAS). Vero and HeLa cells were maintained in minimal essential medium (JRH Biosciences) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, nonessential amino acids, and antibiotics. Madin-Darby bovine kidney (MDBK) cells were grown in minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, nonessential amino acids, and antibiotics.

Viral RNA isolation and amplification by RT-PCR. Supernatant from Vero cells infected with bPIV3 at a multiplicity of infection (MOI) of 0.2 50% tissue culture infective dose (TCID$_{50}$) was harvested 2 days postinfection and centrifuged in an SW28 rotor for 90 min at 25,000 rpm at 4°C. The virus pellet was resuspended in 0.5 ml of phosphate-buffered saline. Total RNA was isolated from this virus preparation by using the RNA STAT-50 LS Reagent (Tel-Test, Inc.). Viral cDNA was prepared using Superscript reverse transcriptase (Gibco/BRL) and an oligonucleotide that was complementary to the 3' end of the viral RNA (5' ACCAAACAAGAGAAGAGACTTGC 3').

The viral genomic cDNA was amplified by PCR using Pfu polymerase (Stratagene). Oligonucleotides complementary to the sense and antisense sequences of the bPIV3 genome were designed based on sequence information available in GenBank (accession nos. D84095, Y00114, Y00115, U31671). Using these specific oligonucleotides, four overlapping PCR fragments harboring the entire bPIV3 genome were produced (nt 1 to 6066, 2149 to 9230, 6047 to 11890, and 9169 to 15456) (Fig. 1A). The oligonucleotide complementary to the 3' end of the bPIV3 genome also contained a SacII site as well as a T7 RNA polymerase promoter followed by two G residues. The four PCR fragments were introduced into plasmid vectors employing the Zero Blunt PCR cloning kit (Invitrogen). The resulting plasmids harboring the four fragments constituting the bPIV3 genome were sequenced to ascertain that the open reading frames of the viral genes were intact.

Cloning of the NP, P and L genes of bPIV3 into a pCITE expression vector. In order to construct the NP/pCITE plasmid, two oligonucleotides complementary to the 5' and 3' end of the viral NP gene and harboring an NcoI site and BamHI site, respectively, were employed to amplify a 1.68-kb DNA fragment (nt 111 to 1786) expressing the NP gene. The PCR fragment was digested with restriction endonucleases NcoI and BamHI and inserted into the pCITE vector digested with NcoI and BamHI.

For the P/pCITE plasmid, a 1.9-kb DNA fragment encompassing the P gene (from nt 1784 to 3700) was prepared by PCR using two oligonucleotides containing an NcoI site and XhoI site, respectively. The P gene fragment was digested with NcoI and XhoI and ligated into the NcoI-BamHI sites of the pCITE vector.

The cloning of the L/pCITE plasmid was accomplished in three steps. First, the hepatitis delta ribozyme (RZ) (25) sequences linked to T7 termination signals and flanked by a BssHII site and a NotI site were positioned downstream of the trailer of bPIV3 by using PCR and overlapping oligonucleotides annealing to both the RZ and bPIV3 trailer sequences. A previously described plasmid (pRSV) served as the PCR template for obtaining the RZ and T7 termination sequences (13). The resulting 0.4-kb PCR fragment containing the RZ and bPIV3 L gene and trailer sequences was digested with EcoRI (nt 15262) and NotI and inserted into pCITE at the EcoRI-NotI sites to generate the complete DNA sequences of the 3' end of the virus. Second, a plasmid harboring the bPIV3 sequences from nt 9169 to 15456 was digested with NdeI and EcoRI. The resulting 5.3-kb DNA fragment (nt 9957 to 15262) was introduced into the RZ/pCITE plasmid generated in the previous step. Third, a 1.3-kb PCR fragment harboring the 5' end of the L gene from nt 8639 to 9957 was digested with

NcoI and NdeI and inserted into the plasmid bearing the rest of the polymerase gene of bPIV3 to reconstruct the complete L gene.

Construction of the full-length bPIV3 cDNA. The plasmid expressing the bPIV3 antigenome was assembled in a stepwise fashion. The first step involved the cloning of a 3.32-kb DNA fragment (nt 5194 to 8509) digested with BstBI and PstI into a modified pUC19 plasmid harboring a custom-designed polycloning site containing all restriction enzyme sites required for construction of the bPIV3 cDNA. Next, a 2.58-kb DNA fragment (nt 8509 to 11087) digested with PstI and SacI was introduced into the plasmid described for the first step such that it contained viral sequences from nt 5194 to 11087. In the third step, the plasmid harboring the sequences from nt 5194 to 11087 was treated with BstBI and SacI in order to isolate the 5.9-kb DNA fragment. This fragment was introduced into the plasmid harboring the fragment from nt 1 to 6066 at the BstBI and SacI sites. The resulting plasmid contained viral DNA sequences from nt 1 to 11087. Finally, L/pCITE was digested with SacI and BssHII to release a 4.6-kb fragment harboring viral sequences from nt 11087 to 15456 linked to the hepatitis delta RZ. This fragment was introduced into the plasmid containing nt 1 to 11087 of bPIV3, resulting in a full-length clone of the bPIV3 genome. By use of the QuikChange mutagenesis kit (Stratagene), an XhoI restriction enzyme site was introduced into the bPIV3 genome at nt 6457 to serve as a genetic marker for positive identification of the recombinant genome.

Construction of the full-length bovine/human PIV3 cDNA. By use of the QuikChange mutagenesis kit (Stratagene), an NheI site was introduced at nt 5042 in the M/F intergenic region and a SalI site was introduced at nt 8530 in the HN/L intergenic region in the full-length bPIV3 plasmid to generate the bPIV3/N/S construct. RNA was isolated from Vero cells infected with hPIV3 (Texas/12084/1983), and the F and HN genes of hPIV3 were amplified by RT-PCR using oligonucleotides that annealed to the 5' and 3' ends of this region. The 5' and 3' oligonucleotides contained an NheI site and a SalI site, respectively. The resulting 3.5-kb PCR fragment was digested with NheI and SalI and was ligated into the bPIV3/N/S plasmid restricted with NheI and SalI to make a full-length chimeric bovine/human PIV3 construct. The cloning junctions of the hPIV3 F and HN gene insertion and the hPIV3 F and HN gene sequences were confirmed by DNA sequence analysis.

Transfection of infectious full-length virus antigenome-containing plasmids. HeLa cells (80% confluent) were infected with vaccinia virus MVA-T7 at an MOI of 4. One hour postinfection, the full-length anti-genomic bPIV3 or the bovine/human PIV3 plasmids (4 μg) were transfected into the MVA-T7-infected HeLa cells together with NP/pCITE (0.4 μg), P/pCITE (0.4 μg), and L/pCITE (0.2 μg) expression plasmids in the presence of 9 μl of LipofecTACE (Gibco/BRL) in 200 μl of Opti-MEM. Forty-eight hours posttransfection, the passage 0 (P0) cells and media were harvested together and subjected to one freeze-thaw cycle. The resulting P0 cell lysate was then used to infect fresh HeLa cell monolayers in the presence of 40 μg of 1-β-D-arabinofuranosylcytosine/μl, a replication inhibitor of vaccinia virus, to generate a P1 virus stock. The media and cells from this infection cycle were harvested and freeze thawed once, and the presence of bPIV3 infection was confirmed by immunostaining of virus plaques using PIV3-specific antiserum or hPIV3-specific antiserum (855-2; Chemicon International).

Recombinant virus stock preparation. The P1 recombinant PIV3 virus stocks were harvested from cells infected with P0. P0 virus stock was obtained from transfected cells. Following three cycles of plaque purification in MDBK or Vero cells, high-titer P2 virus stocks were prepared in Vero cells and stored at −80°C.

Genotyping of r-bPIV3 and bovine/human PIV3. Viral RNA of r-bPIV3, bovine/human PIV3, bPIV3, or hPIV3 was isolated from infected Vero cells using RNA STAT-50 LS Reagent. To verify the genotype of r-bPIV3, the viral cDNA of r-bPIV3 and bPIV3 starting at nt 4500 was prepared using Superscript reverse transcriptase. DNA fragments encompassing the region from nt 5660 to 7447 were amplified by PCR, resulting in a 1.7-kb PCR product. The PCR products were digested with XhoI and separated on a 1% agarose gel. The cDNA of bovine/human PIV3, bPIV3, and hPIV3 was initiated from nt 4500, and PCR fragments from nt 5255 to 6255 (in the F gene) and nt 9075 to 10469 (in the L gene) were amplified. The F gene PCR fragments were digested with SacI or BglII, and the L gene fragments were cleaved with PvuII or BamHI. The DNA fragments were separated on a 1% agarose gel.

Growth curves. Vero or MDBK cells were grown to 90% confluence and infected at an MOI of 0.01 with bPIV3, r-bPIV3, hPIV3, or bovine/human PIV3. The infected monolayers were incubated at 37, 39, and 40°C. At 12, 24, 48, 72, and 96 h postinfection, cells and media were harvested together and stored at −80°C. Virus titers for each time point harvest were determined by TCID$_{50}$ assay in MDBK cells.

Efficiency of plaque formation assay. Plaque assays were carried out on Vero cells with bPIV3, r-bPIV3, hPIV3, or bovine/human PIV3. The infected monolayers were incubated at 35, 37, 38, 39, 39.5, and 40°C. Four days postinfection, the infected monolayers were immunostained using bPIV3- or hPIV3-specific antisera. The plaques were quantified, and titers were determined at the different temperatures to identify the shutoff temperatures of the viruses.

Western and immunoprecipitation analyses. For Western analysis, Vero cells were infected with bPIV3, hPIV3, r-bPIV3, or bovine/human PIV3 at an MOI of 0.01. Forty-eight hours postinfection, the media were removed, the cells were washed once with cold phosphate-buffered saline, and the proteins were ex-

TABLE 1. Nucleotide alterations in the r-bPIV3 genome

Nucleotide position in the virus genome	Virus gene	Nucleotide		Amino acid change
		bPIV3 (Kansas/15626/84)	r-bPIV3	
6460	F gene	U	G	None
6463	F gene	U	C	None
11946	L gene	U	C	Ile→Val
15354	L gene	A	U	None

tracted with lysis buffer. The cell lysate was fractionated on a 10% protein gel, transferred onto a nylon membrane, and probed with PIV3-specific antisera. The protein antibody complexes were visualized by chemiluminescence (Amersham).

For immunoprecipitation, Vero cells were infected with bPIV3, hPIV3, r-bPIV3, or bovine/human PIV3 at an MOI of 0.5. Twenty-four hours postinfection, the cells were washed once with Dulbecco's modified Eagle's medium (DME) without cysteine and methionine (ICN) and incubated in 2 ml of the same media for 30 min. The medium was removed, and 0.5 ml of DME (lacking cysteine and methionine) containing 100 μCi of [^{35}S]-Pro-Mix (Amersham) was added to the cells. The infected cells were incubated in the presence of ^{35}S isotopes for 5 h at 37°C. The media were removed, and the infected cells were lysed in 0.3 M radioimmunoprecipitation assay buffer containing protease inhibitors. The cell lysate was incubated with PIV3-specific antiserum and bound to anti-mouse immunoglobulin G-agarose (Sigma). After washing three times with 0.5 M radioimmunoprecipitation assay buffer, the samples were fractionated on a 12% protein gel. The gel was dried and exposed to X-ray film.

Small-animal studies. Five-week-old Syrian golden hamsters (four animals per group) were infected with 5 × 10^5 PFU of bPIV3, r-bPIV3, hPIV3, bovine/human PIV3, or Opti-MEM placebo in a 100-μl volume. The five different groups were maintained separately in microisolator cages. Four days postinfection, the nasal turbinates and lungs of the animals were harvested, homogenized, and stored at −80°C. The titers of virus present in the tissues were determined by TCID$_{50}$ assays in MDBK cells. Virus infection was confirmed by hemadsorption with guinea pig or chicken red blood cells. For the challenge studies, eight hamsters per group were initially infected with 5 × 10^5 PFU of bPIV3, r-bPIV3, hPIV3, bovine/human PIV3, or placebo. Four animals were sacrificed per group and analyzed for virus replication as described above. The rest of the animals were maintained until day 21. On day 21, the animals were challenged with 10^6 PFU of wt hPIV3 (Washington/47885/57) per animal in a 100-μl volume. Four days postchallenge, the animals were sacrificed and the lungs and nasal turbinates were harvested. These tissues were then analyzed for the presence of hPIV3 by TCID$_{50}$ and hemadsorption assays.

HAI assays. Serum samples collected from the hamsters prior to infection and at day 21 postinfection were analyzed in a hemagglutination inhibition (HAI) assay. Briefly, the serum samples were treated with receptor-destroying enzyme (DENKA Seiken Co.). bPIV3 and hPIV3 were added to twofold-serially diluted hamster serum. Finally, guinea pig red blood cells were added, and hemagglutination was allowed to occur at room temperature.

RESULTS

Construction of the full-length bPIV3 cDNA and rescue of r-bPIV3. An infectious full-length antigenomic cDNA of bPIV3 was constructed by RT-PCR amplification of virion genomic RNA to produce four overlapping 4- to 5-kb DNA segments, which were assembled in sequential cloning steps as illustrated in Fig. 1A. The assembled viral cDNA was sequenced to verify the existence of intact open reading frames for each of the viral genes. The sequence obtained for the r-bPIV3 genome was compared to that for bPIV3 (Kansas/15626/84) available in GenBank (accession no. AF178654) (1). Four nucleotides were identified in r-bPIV3 that differed from those of the bPIV3 RNA genome (Table 1). Two nucleotide substitutions of r-bPIV3 were present in the F gene (U→G and U→C) (Table 1), representing the introduction of the genetic marker, an XhoI restriction enzyme site at nt 6457. These nucleotide alterations were engineered in a manner so as not to change the amino acid sequence of the F protein of r-bPIV3. Interestingly, the third nucleotide change was found in the L gene at nt 11946 of r-bPIV3 (U→C) (Table 1). This substitution in the viral sequence changed an isoleucine residue in the L protein of bPIV3 to a valine in the L protein of r-bPIV3. The

fourth nucleotide change at nt 15354 (A→U) was located in the 3′ untranslated region of the L gene (Table 1). This residue was located 12 nt downstream from the L protein stop codon, but it did not alter the conserved gene stop sequence of the L gene. The experiments described below were carried out to evaluate the effect of these sequence changes in the r-bPIV3 genome on virus replication in vitro and in vivo.

The functional integrity of the bPIV3 cDNA was tested by experiments in which infectious virus was rescued from transfected anti-genomic cDNA in the presence of the required expression plasmids NP, P, and L. The plaques of the recovered r-bPIV3 were immunostained using an antiserum specific for PIV3. Interestingly, bPIV3 virus recovery was very efficient in HeLa cells. The P1 virus stocks generated titers of 2.4 × 10^6 PFU/ml. A high-yield virus rescue system is advantageous for a virus vector because the introduction of foreign genes will most likely reduce the rescue efficiency. The plaque sizes of r-bPIV3 and bPIV3 were comparable (data not shown). To verify at the molecular level that the recovered PIV was r-bPIV3, PCR fragments of the F gene of r-bPIV3 were analyzed for the presence of the introduced genetic marker, an XhoI restriction enzyme site (Fig. 2). The 1.7-kb PCR fragment originating from r-bPIV3 was cleaved with XhoI, resulting in the two predicted smaller DNA fragments (Fig. 2, lane 3). As a control, the same region of the bPIV3 RNA was amplified by PCR. As expected, the bPIV3 PCR fragment remained resistant to XhoI digestion and migrated similarly to the full-length 1.7-kb DNA fragment (Fig. 2, lane 5). Identification of the XhoI site demonstrated that the recovered r-bPIV3 was indeed derived from plasmid DNA containing the full-length bPIV3 genome by reverse genetics. This result showed that the anti-genomic bPIV3 cDNA clone was functional in that it supported the rescue of infectious recombinant viruses.

Construction of the infectious bovine/human PIV3 cDNA. The ability of r-bPIV3 to deliver foreign antigens was evaluated. The F and HN genes of bPIV3 were replaced with those
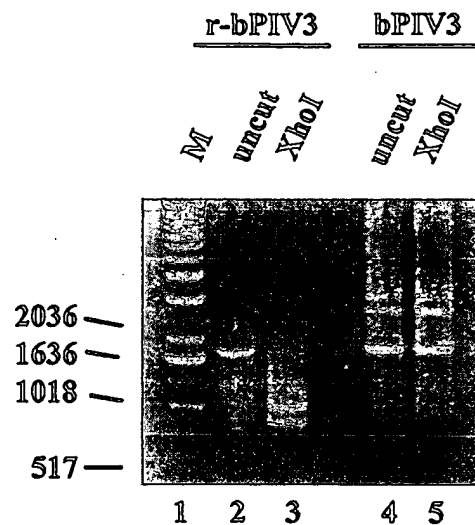

FIG. 2. Identification of the genetic marker in the recovered r-bPIV3 genome. PCR fragments, 1.7 kb in length, were amplified by RT-PCR from cells infected with r-bPIV3 and bPIV3. The amplified region contained viral sequences from nt 5660 to 7447 in the F gene. The digested and undigested fragments were analyzed on a 1% agarose gel. Lanes 2 and 4 show the undigested PCR fragments of r-bPIV3 and bPIV3, respectively. Lanes 3 and 5 depict the DNA fragments of r-bPIV3 and bPIV3, respectively, treated with XhoI. The base pair positions of a DNA size marker (M) in lane 1 are indicated.

of hPIV3 because the surface glycoproteins F and HN of bPIV3 and hPIV3 are 77 and 80% identical, respectively. The high degree of conservation of the bPIV3 and hPIV3 surface glycoproteins indicated that the likelihood of recovery of this chimeric bovine/human PIV3 was high, especially since the F and HN proteins are essential components of a viable and infectious PIV particle. This chimeric bovine/human PIV3 was expected to express the hPIV3-neutralizing antigens, utilizing the bPIV3 replication machinery. Further, bovine/human PIV3 was expected to be genetically stable because both the F and HN proteins are integral virion components.

A comparison of the gene stop and gene start sequences located in the F/HN intergenic regions of both bPIV3 and hPIV3 showed that they were highly conserved. Only 4 nt of bPIV3, 2 nt in the F stop sequence, and 2 nt in the HN start sequence differed from that of hPIV3 (Fig. 1B). This conservation was predicted to enable recognition of the hPIV3 gene stop and gene start sequences by the bPIV3 polymerase (Fig. 1B). Interestingly, the dissimilar nucleotides were located in regions that permitted variability, while all of the other positions were identical (17). Thus, it was not necessary to change nucleotides in the gene stop or gene start sequence of the F/HN intergenic region of the hPIV3 (Texas/12084/1983) F and HN gene fragment. The F and HN genes of bPIV3 were excised from the plasmid bPIV3/N/S and replaced with those of hPIV3 (Fig. 1B). The genome of bovine/human PIV3 contained the same two nucleotide substitutions in the L gene that were present in r-bPIV3. The functionality of the full-length chimeric bovine/human PIV3 plasmid DNA was tested by virus recovery using reverse genetics.

Rescue of bovine/human PIV3 and confirmation of its chimeric genotype. The bovine/human PIV3 antigenomic DNA expressed the F and HN genes of hPIV3 in place of those of bPIV3. The full-length DNA of chimeric bovine/human PIV3 was transfected into HeLa cells along with supporting plasmids expressing bPIV3 NP, P, and L proteins. The rescue efficiency of bovine/human PIV3 was high—the P1 virus stock had a titer of 10⁴ PFU/ml, and the plaques had a size similar to that of the plaques produced by its parents, r-bPIV3 and hPIV3 (data not shown). The chimeric properties of the P2 stock of bovine/human PIV3 were verified by three criteria. First, a plaque assay of the P2 virus stock was immunostained using an antiserum specific for the hPIV3 HN protein. Second, a region of the F gene of bovine/human PIV3, bPIV3, and hPIV3 was amplified by PCR. The resulting PCR products were treated with SacI and BglII, both of which were expected to cleave the hPIV3 F gene but not the analogous F gene of bPIV3. Only the DNA fragments containing hPIV3 sequences were cut by SacI and BglII, while bPIV3 sequence-containing fragments were not cleaved (Fig. 3A, compare lanes 3, 4, 7, and 8 with lanes 11 and 12). In addition, a region of the L gene of bovine/human PIV3, bPIV3, and hPIV3 was amplified by PCR to demonstrate that it harbored restriction enzyme sites present only in the bPIV3 genome (Fig. 3B). The resulting PCR products were digested with BamHI or PvuII. Upon digestion with these enzymes, the DNA fragments derived from bovine/human PIV3 or bPIV3 showed similar restriction patterns (Fig. 3B, lanes 3, 4, 11, and 12). However, the PCR fragments originating from hPIV3 were not cleaved in the presence of either PvuII or BamHI (Fig. 3B, lanes 7 and 8). These results showed that the L polymerase fragment of bovine/human PIV3 was derived from bPIV3. Genotyping of the bovine/human PIV3 demonstrated that the F gene of chimeric bovine/human PIV3 was derived from hPIV3, whereas the polymerase gene originated from bPIV3. Third, the entire F and HN genes of the chimeric bovine/human PIV3 genome and the flanking inter-

A. PCR fragments of the F gene
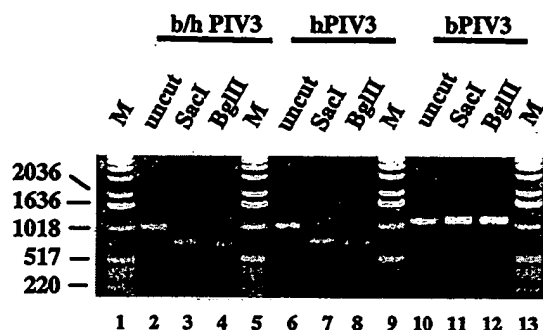
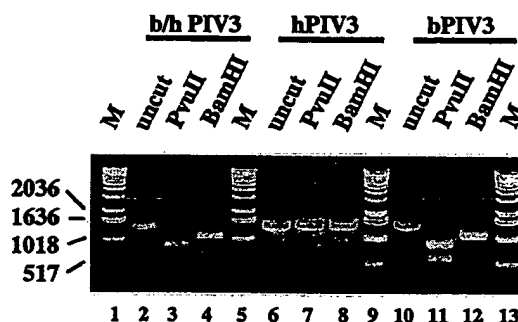## B. PCR fragments of the L gene

FIG. 3. Genotyping of bovine/human PIV3 to confirm its chimeric RNA genome. (A) PCR fragments (1.1 kb) of the F gene (from nt 5255 to 6255) derived from bovine/human PIV3, hPIV3, and bPIV3 were analyzed for the presence of SacI and BglII restriction enzyme sites unique to the hPIV3 sequences (lanes 3, 4, 7, 8, 11, and 12). The full-length PCR fragments are shown in lanes 2, 6, and 10. Lanes 1, 5, 9, and 13 represent the DNA size marker (M) (in base pairs). (B) PCR fragments of the L gene were tested for the presence of PvuII and BamHI restriction enzyme sites unique to the bPIV3 genome. PCR fragments were prepared from Vero cells infected with bovine/human PIV3, hPIV3, or bPIV3. The 1.4-kb PCR products were digested with PvuII or BamHI (lanes 3, 4, 7, 8, 11, and 12). Undigested 1.4-kb DNA fragments are shown in lanes 2, 6, and 10. Lanes 1, 5, 9, and 13 represent the DNA size marker (M) (in base pairs). The DNA fragments were separated on a 1% agarose gel.

genic regions were amplified by PCR, and the resulting PCR products were sequenced (Fig. 4). The nucleotide sequence of bovine/human PIV3 was confirmed to contain the hPIV3 F and HN gene sequences. However, a single amino acid substitution at residue 459 in the F protein from a glutamic acid to a glycine was identified. Furthermore, the M/F and HN/L intergenic regions of bovine/human PIV3 displayed the introduced restriction enzyme sites NheI and SalI at the bovine/human junctions. Other unexpected nucleotide alterations in the F and HN genes of the hPIV3 insert were not observed. These data demonstrated that the virus rescued was a chimeric bovine/human PIV3.

In vitro characterization of r-bPIV3 and bovine/human PIV3 in Vero cells. The multicycle replication of bPIV3, r-bPIV3, bovine/human PIV3, and hPIV3 was studied in Vero cells (Fig. 5). Briefly, subconfluent Vero cells were infected at an MOI of 0.01 and incubated at 37, 39, and 40°C. Time points were taken

A. M/F junction region from nt 5031 to 5086:

bPIV3/N/S CCAAAACAGAG**G**C**U**AG**C**AGACAAAGAGCACCAGAU*AUG*ACCAUCACAACC

b/h PIV3 CCAAAACAGAG**GCUAGC**CAACAAGCACUGAACAC*AAUG*CCAACUUCAAUA

hPIV3   CAACAAGCACUGAACAC*AAUG*CCAACUUCAAUA

          M/F intergenic region   NheI   start   F gene

B. HN/L junction region from nt 8501 to 8552:

bPIV3/N/S CCAAAAAACUGCAGC*UAA*AUUGA**GUCGA**CCAUAUCGGAUGCAAGAU

b/h PIV3 CCAAAAAGCUGCAGU*UAA*UCAUA**GUCGA**CCAUAUCGGAUGCAAGAU

hPIV3   CCAAAAAGCUGCAGU*UAA*UCAUA

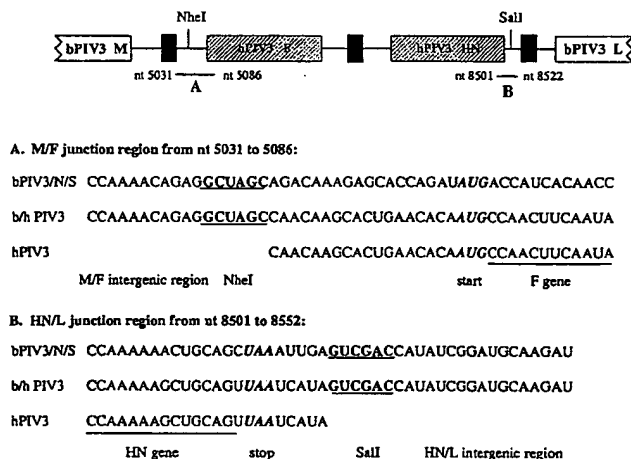   HN gene   stop   SalI   HN/L intergenic region

FIG. 4. A diagram of the chimeric M/F and HN/L intergenic junctions of bovine/human PIV3 showing the introduced nucleotide changes. The gene stop and gene start sequences are indicated by black rectangles. The M/F and HN/L intergenic junctions as well as the hPIV3 F and HN genes of the bovine/human PIV3 were sequenced and aligned with the homologous regions of bPIV3 and hPIV3. (A) The M/F intergenic junctions from nt 5031 to 5086 of bPIV3/N/S, bovine/human PIV3, and hPIV3 are shown. The introduced NheI site is indicated by underlined bold letters. The AUG translation start codon of the F gene is noted in bold italics. The first nucleotides encoding the F protein are underlined. The 18 nt of bovine/human PIV3 preceding the AUG start codon are derived from hPIV3. In contrast, the sequences of bovine/human PIV3 preceding the NheI restriction enzyme site match the bPIV3/N/S sequences. (B) The nucleotide sequences of the HN/L intergenic junctions from nt 8501 to 8552 of bPIV3/N/S, bovine/human PIV3, and hPIV3 are shown. The introduced SalI site is indicated by underlined bold letters. The UAA translation stop codon is denoted by bold italic letters. Nucleotides encoding the C-terminal amino acids of HN are underlined. The region of bovine/human PIV3 preceding the SalI site is identical to that of hPIV3, while the region of bovine/human PIV3 following the SalI site matches that of bPIV3/N/S.

by harvesting the cells as well as the media at 12, 24, 48, 72, and 96 h postinfection. The amount of virus at each time point was then determined by TCID$_{50}$ assays.

At 37°C, r-bPIV3 replicated as well as bPIV3 in Vero cells. Peak titers of 8.6 and 8.9 log$_{10}$ TCID$_{50}$ were observed for r-bPIV3 and bPIV3, respectively (Fig. 5A). The onset of r-bPIV3 replication was slightly delayed at 39°C, compared to that of bPIV3 (Fig. 5B). The peak titer of r-bPIV3 at 39°C in Vero cells 48 h postinfection was reduced consistently by 2 log$_{10}$ from that of bPIV3 (Fig. 5B). This temperature sensitivity of r-bPIV3 was more obvious at 40°C. r-bPIV3 displayed dramatic 4.8 and 3.5 log$_{10}$ reductions at 40°C from the peak titers at 37 and 39°C, respectively (Fig. 5A to C). Surprisingly, bPIV3 was also temperature sensitive at 40°C and displayed 2.2 and 1.4 log$_{10}$ reductions at 40°C from the peak titers at 37 and 39°C, respectively (Fig. 5A to C). However, the Vero cells appeared viable at 40°C. A similar kinetics of virus replication for r-bPIV3 and bPIV3 was observed in MDBK cells (data not shown). In summary, r-bPIV3 displayed a delayed kinetics for virus growth and a temperature-sensitive phenotype in Vero and MDBK cells at 39 and 40°C. These data suggest that the four genetic alterations present in r-bPIV3 may have contributed to its temperature-sensitive phenotype.

In order to assess the impact of F and HN gene replacement on virus replication, the replication kinetics of bovine/human PIV3 were studied. At 37°C, the bovine/human PIV3 replicated nearly as well as r-bPIV3 and hPIV3, the parental virus strains (Fig. 5A). Interestingly, replication of bovine/human PIV3 was impaired to a greater extent at 39°C than at 37°C (Fig. 5A and B). The peak titers of bovine/human PIV3 at 39°C

were decreased by 1.8 and 2.1 log$_{10}$ from those of r-bPIV3 and hPIV3, respectively (Fig. 5B). At 48 h postinfection, bovine/human PIV3 had reached its peak of growth and leveled off, while titers of r-bPIV3 and hPIV3 continued to increase (Fig. 5B). At 40°C, bovine/human PIV3 displayed a dramatic decrease in replication in Vero cells. The titers of bovine/human PIV3 were reduced 4.3 log$_{10}$ at 40°C from those observed at 37°C (Fig. 5A and 5C). Indeed, the kinetics of virus replication of chimeric bovine/human PIV3 at 40°C resembled that of its r-bPIV3 parent (Fig. 5C). In contrast, the wt parent hPIV3 showed levels of replication only slightly decreased at 40°C from those at 37°C (Fig. 5A and 5C). The replacement of the bPIV3 F and HN genes with those of hPIV3 resulted in a temperature-sensitive chimeric bovine/human PIV3 that replicated to high titers at 37°C in Vero cells.

r-bPIV3 and bovine/human PIV3 displayed temperature-sensitive phenotypes in the multicycle growth curves. To identify the specific shutoff temperatures of r-bPIV3 and bovine/human PIV3, plaque assays at 35, 37, 38, 39, 39.5, and 40°C were performed (Table 2). Both recombinant viruses, r-bPIV3 and bovine/human PIV3, displayed a shutoff temperature of 39.5°C, at which there was a >100-fold reduction of plaquing efficiency (Table 2). r-bPIV3 and bovine/human PIV3 possess two mutations in the polymerase gene, one of which resulted in an amino acid change from an isoleucine to a valine. Amino acid changes in viral polymerases often produce viruses that harbor temperature-sensitive phenotypes (29). Most likely, the
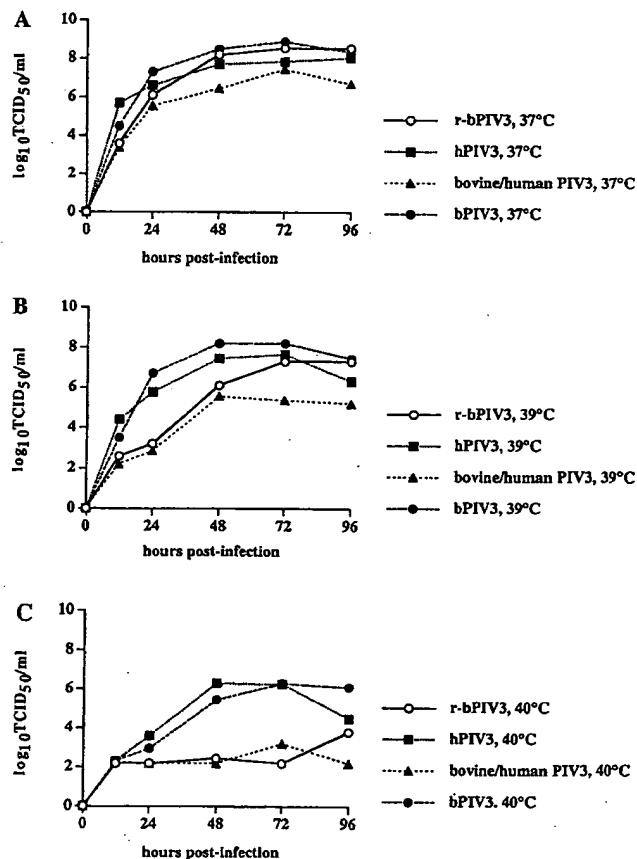

FIG. 5. Multicycle growth curves of bovine/human PIV3, r-bPIV3, bPIV3, and hPIV3 were carried out at 37, 39, and 40°C in Vero cells. Collection times were at 12, 24, 48, 72, and 96 h postinfection. The samples were analyzed for virus titers by TCID$_{50}$ assays in MDBK cells.

TABLE 2. Determination of the shutoff temperature for
r-bPIV3 and bovine/human PIV3

Virus	Mean virus titer ($\log_{10}$ PFU/ml) at indicated temp (°C)[a]						Temperature sensitivity phenotype
	35	37	38	39	39.5	40	
bPIV3	9.0	8.9	9.1	8.9	8.9	8.8	−
r-bPIV3	8.9	8.6	8.4	7.4	6.9	4.7	+
hPIV3	7.6	7.7	7.8	7.5	7.6	7.6	−
Bovine/human PIV3	7.9	7.8	7.7	6.4	5.7	3.8	+

[a] Plaques were enumerated by immunoperoxidase staining after incubation for 4 days at the indicated temperature. The average of three experiments is presented. Underlined values represent the restrictive temperature at which there was a 100-fold reduction of plaquing efficiency, which is defined as the shutoff temperature of plaque formation.

polymerase mutation causing the amino acid substitution and present in both r-bPIV3 and bovine/human PIV3 was responsible for their temperature-sensitive phenotypes.

Expression of the introduced F and HN hPIV3 genes of bovine/human PIV3 was studied by Western blot analysis and by immunoprecipitation using PIV3 antisera and bovine/human PIV3-infected cell lysates (data not shown). The level of protein expression of the F and HN proteins was proportional to that of the other viral proteins and similar to that observed for bPIV3 and wt hPIV3. There were no electrophoretic mobility differences between the F and HN proteins of bovine/human PIV3 and hPIV3 (data not shown).

Replication of r-bPIV3 and bovine/human PIV3 in the upper and lower respiratory tracts of hamsters. Syrian golden hamsters have been shown to be permissive for infection by hPIV3 and have been employed as a model for attenuation (8). Therefore, the abilities of r-bPIV3 and bovine/human PIV3 to replicate in vivo were studied in Syrian golden hamsters. Both bPIV3 and hPIV3 attained high titers (approximately 10^5 $TCID_{50}$/g of tissue) in the lungs and nasal turbinates of hamsters (Table 3). In contrast, r-bPIV3 attained a titer in the lungs of hamsters reduced by 2 $\log_{10}$ ($10^{2.9}$ $TCID_{50}$/g of tissue) from that of bPIV3 (Table 3). These data suggest that the nucleotide changes introduced during the cloning process of the r-bPIV3 genome may be responsible for the decreased replication of r-bPIV3 in the lungs of hamsters. In summary, r-bPIV3 was attenuated for replication in the lungs of Syrian golden hamsters.

Bovine/human PIV3's ability to replicate in Syrian golden hamsters was also analyzed. Interestingly, chimeric bovine/human PIV3 replicated efficiently in the nasal turbinates of hamsters ($10^{4.7}$ $TCID_{50}$/g of tissue) (Table 3). The virus titers observed in the nasal turbinates of hamsters were close to

those of hPIV3 and r-bPIV3, the parental virus strains (Table 3). Replication of bovine/human PIV3 in the lungs of hamsters was reduced by 1 $\log_{10}$ from that of hPIV3 (Table 3). Bovine/human PIV3 displayed an intermediate in vivo phenotype for replication in the lungs of hamsters—it replicated not as well as hPIV3 but to slightly higher levels than did r-bPIV3. The introduction of the hPIV3 F and HN genes into the r-bPIV3 genome improved the ability of bovine/human PIV3 to replicate in the lungs of hamsters. However, bovine/human PIV3 was still attenuated for replication in the lungs of Syrian golden hamsters, compared to hPIV3 or bPIV3. The observed attenuation phenotype of bovine/human PIV3 may be determined by the two nucleotide alterations in the L gene, which led to a decreased compatibility of the combined human and bovine genomes to form functional virions.

Immunization with chimeric bovine/human PIV3 produced antibodies specific for hPIV3. The levels of hPIV3-specific antibodies produced in the parainfluenza-infected hamsters were measured by performing HAI assays on hamster serum collected 21 days postimmunization. To determine whether the antiserum showed specificity for either hPIV3 or bPIV3, both viruses were tested in the HAI assay. Surprisingly, both bPIV3- and r-bPIV3-immunized animals displayed low HAI antiserum titers for both bPIV3 (1:16 and 1:12, respectively) and hPIV3 (1:8 and 1:12, respectively) (Table 4). Antiserum from animals infected with hPIV3 showed a strong HAI response to hPIV3 (1:128) and a much weaker response to bPIV3 (1:10) (Table 4). The antibody response of animals infected with bovine/human PIV3 was identical to that obtained with hPIV3. The HAI titers for hPIV3 were high (1:128) and lower for bPIV3 (1:6.7) (Table 4). Serum samples from animals given a placebo displayed a background signal of <1:4 (Table 4). These data show that bovine/human PIV3 expressing the hPIV3 surface glycoproteins F and HN elicited an immune response identical to that elicited by wt hPIV3 and specific for hPIV3. These data suggest that bovine/human PIV3 was an attenuated virus that elicited a strong immune response in Syrian golden hamsters producing hPIV3-specific antibodies.

Bovine/human PIV3 protected hamsters from challenge with hPIV3. Studies were carried out to determine whether the recombinant viruses could protect Syrian golden hamsters from challenge with wt hPIV3 (Washington/47885/57 strain). Twenty-one days after hamsters were immunized with hPIV3, bPIV3, r-bPIV3, or bovine/human PIV3, the animals were challenged by intranasal inoculation with 10^6 PFU of hPIV3. Four days postchallenge, the hamsters were sacrificed and their nasal turbinates and lungs were harvested. Virus titers were determined by $TCID_{50}$ assay. All of the immunizing viruses (bPIV3, r-bPIV3, hPIV3, and bovine/human PIV3) provided complete protection from challenge with wt hPIV3 (Table 4). Less than 1.3 $\log_{10}$ $TCID_{50}$/g of tissue, the limit of detection for this assay, was observed for all samples irrespective of whether they were derived from the nasal turbinates or lungs. Only unimmunized animals displayed high levels of hPIV3 replication in both the lungs and nasal turbinates ($\sim 10^5$ $TCID_{50}$/g of tissue) (Table 4). PIV3 infection was confirmed by hemadsorption of the $TCID_{50}$ plates with guinea pig red blood cells. Hemadsorption with red blood cells, a highly sensitive assay for PIV3 infectivity, failed to detect challenge virus in tissues obtained from previously immunized hamsters. Only animals that received the placebo inoculum showed hemadsorbed red blood cells. These data further show that the single amino acid substitution from a glutamic acid residue to a glycine in the F protein of bovine/human PIV3 did not affect immunogenicity of the surface glycoproteins. In summary, infection of Syrian golden hamsters with r-bPIV3 and bovine/

TABLE 3. Replication of bPIV3, r-bPIV3, hPIV3 and
bovine/human PIV3 in the upper and lower respiratory tracts
of hamsters

Virus[a]	Mean virus titer ± SE on day 4 postinfection ($\log_{10}$ $TCID_{50}$/g of tissue)	
	Nasal turbinates	Lungs
bPIV3	5.5 ± 0.0	5.2 ± 0.0
r-bPIV3	5.0 ± 0.4	2.9 ± 0.5
wt hPIV3	4.9 ± 0.1	4.8 ± 0.1
Bovine/human PIV3	4.7 ± 0.1	3.7 ± 0.4
Placebo	<1	<1

[a] Groups of four hamsters were inoculated intranasally with 5×10^5 PFU of indicated virus.

TABLE 4. r-bPIV3 and bovine/human PIV3 are immunogenic and protect hamsters upon challenge with wt hPIV3

Immunizing virus[a]	Response to challenge: mean virus titer ± SE ($\log_{10}$ $TCID_{50}$/g of tissue)[b]		Serum HAI antibody titer (reciprocal mean $\log_2$)[c]	
	Nasal turbinates	Lungs	bPIV3	wt hPIV3
bPIV3	<1.3	<1.1	16.0 ± 0.0	8.0 ± 0.0
r-bPIV3	<1.2	<1.0	12.0 ± 0.0	12.0 ± 0.0
wt hPIV3	<1.2	<1.1	10.0 ± 0.2	128.0 ± 0.0
Bovine/human PIV3	<1.2	<1.0	6.7 ± 0.1	128.0 ± 0.0
Placebo	5.5 ± 0.2	5.1 ± 0.4	<4.0 ± 0.0	<4.0 ± 0.0

[a] Virus used to immunize groups of four hamsters on day 0.

[b] On day 28, all hamsters were bled to determine the serum HAI antibody titers and were challenged with 10^6 PFU of wt hPIV3. Four days postchallenge, the lungs and nasal turbinates of the animals were harvested.

[c] The HAI data are derived from at least two independent experiments.

human PIV3 resulted in an immune response that completely protected the animals from subsequent infection by wt hPIV3.

DISCUSSION

In this study, the RNA genome of a negative-strand virus, bPIV3, was cloned by RT-PCR and assembled into a full-length infectious anti-genomic DNA. Recombinant bPIV3 was recovered from plasmid DNA by reverse genetics. Since bPIV3 exhibited a host cell-restricted replication phenotype in humans and did not cause disease in infants or children, bPIV3 was tested as a virus vector expressing the surface glycoproteins of hPIV3. The resulting bovine/human PIV3 was shown to contain the F and HN genes of hPIV3 by sequencing the surface glycoprotein sequences. Expression of the hPIV3 F and HN proteins of the chimeric PIV3 was confirmed in vitro by Western and immunoprecipitation analyses as well as in vivo by carrying out challenge studies with wt hPIV3 in Syrian golden hamsters. The animals vaccinated with bovine/human PIV3 were completely protected from infection with wt hPIV3 and displayed an HAI antibody response specific for hPIV3. We have thus clearly shown that r-bPIV3 can serve as a vector to express and deliver foreign antigens. This approach can be expanded to express antigens from hPIV1, hPIV2, and other viral or bacterial antigens.

r-bPIV3 was initially characterized for virus replication in vitro and in vivo. Interestingly, r-bPIV3 displayed slightly altered growth characteristics compared to those of bPIV3, from which it was derived. r-bPIV3 displayed a shutoff temperature of 39.5°C. Growth curves showed that r-bPIV3 replicated as well as bPIV3 at 37°C in Vero cells. However, r-bPIV3 displayed virus titers at 39 and 40°C in Vero cells reduced from those of bPIV3. In vivo studies in Syrian golden hamsters showed that r-bPIV3 was attenuated for replication in lungs but not nasal turbinates. In contrast, bPIV3 replicated to high titers in both the lungs and nasal turbinates. The discrepancies in behavior between the bPIV3 and r-bPIV3 suggest that the nucleotide alterations present in the RNA genome of r-bPIV3 rendered it genetically distinct from bPIV3. A comparison of the RNA genomes of r-bPIV3 and the bPIV3 strain from which r-bPIV3 was derived (Kansas/15626/84) revealed four nucleotide changes that were introduced during the cloning process. The nucleotide changes introduced in the F gene are most likely not involved in causing the observed phenotypes of r-bPIV3, because these changes are silent and do not result in amino acid substitutions in the F protein. In contrast, the amino acid change in the L polymerase protein in which an isoleucine was substituted by a valine may be the cause of both the temperature-sensitive and attenuation phenotypes displayed by r-bPIV3. This amino acid change is not located in

regions harboring conserved polymerase motifs (31). Examples of amino acid substitutions in viral polymerases resulting in virus phenotypes in vitro and in vivo have been described before. For example, three single-nucleotide mutations (at nt 11468, 11618, and 13308) in the L polymerase gene of a cold-passage (cp45) hPIV3 were identified that contributed to temperature sensitivity and attenuation of cp45 but not to the cold-adapted growth phenotype. The nucleotide alterations in the cp45 hPIV3 genome resulted in the following amino acid substitutions: Tyr-942→His, Leu-992→Phe, and Thr-1558→Ile (29). The nucleotide change at position 11946 in the r-bPIV3 polymerase gene is present in the same vicinity of the polymerase protein as those observed in cp45 hPIV3 and changes amino acid 1111 from Val to Ile. The nucleotide substitution in the 3' untranslated region of the L gene of r-bPIV3, the fourth nucleotide alteration, may also contribute to the observed phenotypes of r-bPIV3. Untranslated regions of RNA viruses often harbor determinants for viral transcription and viral replication, such as polymerase binding sites or packaging signals (12). Future experiments will determine whether these nucleotide changes are indeed responsible for the observed in vitro and in vivo phenotypes of r-bPIV3.

Despite being attenuated in vivo, r-bPIV3 can protect hamsters very effectively from challenge with wt hPIV3. Curiously, the observed HAI antibody titers of the sera collected from hamsters immunized with bPIV3 or r-bPIV3 were low. This result might suggest a lack of immunogenicity of the administered antigen, in this case bPIV3 or r-bPIV3. However, low HAI titers have been previously observed in both rhesus monkeys and humans immunized with bPIV3 (15, 16, 33). In all cases, both the primates and humans were protected from subsequent challenge with wt hPIV3 (15, 16, 33). van Wyke Coelingh et al. (33) compared the antibody response produced by squirrel monkeys and chimpanzees immunized with bPIV3 and challenged with hPIV3. The antibody titers present in the primate sera were tested by both HAI and neutralization assays. The antibody titers obtained by the different methods were comparable. Thus, it appears that both bPIV3 and r-bPIV3 activate additional immune pathways other than humoral immunity, perhaps by stimulating the T-cell-mediated immune response. Alternatively, the observed immune protection may also be due to immunoglobulin A not detected by the serum HAI assay. Other cases exist where protection from wt viruses was successful despite low HAI serum antibody titers. For example, humans and primates immunized with influenza virus type A and type B displayed low HAI antibody levels but were successfully protected against infection with wt influenza virus strains (3, 19, 20). These results suggest that immunity to PIVs, like immunity to influenza viruses, is not accurately predicted

by serum antibody responses alone but may also involve mucosal and cellular responses.

The attenuation phenotype of bPIV3 observed upon immunization of humans is based on the absence of disease caused by PIV3 in humans. However, the genetic loci of the attenuation determinants of bPIV3 are not yet identified. A recent study by Bailly et al. described an hPIV3 that expressed the NP protein of bPIV3 (2). Interestingly, this chimeric PIV3 was not impaired for virus growth in vitro yet was attenuated in rhesus monkeys. These data suggest that at least some of the attenuation determinants reside in the NP protein of bPIV3. Further, a chimeric bovine/human respiratory syncytial virus (RSV) was evaluated as a live, attenuated vaccine in chimpanzees, a host that is highly permissive for hRSV infection but does not support bRSV replication (4). However, replacing the surface glycoproteins of bRSV with those of hRSV did not result in efficient replication in chimpanzees. These data indicate that the F and G proteins of bRSV were not the major determinants of the host range phenotype. Similarly, we anticipate that the F and HN proteins of bPIV3 will play only a minor role in the host cell-restricted replication observed for bPIV3 in humans. We expect that the major attenuation determinants will be associated with the replication machinery of bPIV3, in particular the polymerase protein complex.

Rescue of chimeric bovine/human PIV3 clearly demonstrated that r-bPIV3 can serve as a vector to express antigens of human PIVs. Interestingly, chimeric bovine/human PIV3 displayed characteristics of both parents, r-bPIV3 and hPIV3. In contrast to hPIV3, bovine/human PIV3 was temperature sensitive at 39°C in Vero cells, displayed a shutoff temperature of 39.5°C, and was attenuated for replication in the lungs of hamsters like r-bPIV3. The observed temperature sensitivity and attenuation in vivo of bovine/human PIV3 may be the result of the single amino acid substitution in the L protein as well as the introduction of the hPIV3 surface glycoproteins. In contrast to r-bPIV3, bovine/human PIV3 induced antibodies in hamsters at levels equal to those observed for wt hPIV3. The HAI antibody titers elicited by both hPIV3 and bovine/human PIV3 are much higher than those elicited by either r-bPIV3 or bPIV3. Similarly, a chimeric human PIV3/PIV1 (r-PIV3-1) that expressed the hPIV1 F and HN proteins in an hPIV3 genetic backbone displayed characteristics from both parental viruses (32). This chimeric virus was trypsin dependent for plaque formation in tissue culture, as is observed for wt hPIV1 but not hPIV3. The r-PIV3-1 also caused extensive cytopathic effects in LLC-MK2 cell monolayers, like hPIV3 but unlike the noncytopathic hPIV1 parental virus (32).

Chimeric bovine/human PIV3 will also be evaluated as a putative hPIV3 vaccine. Evaluation in primates, such as rhesus monkeys, will provide further information on the location of the attenuation determinants. These results will determine whether bovine/human PIV3 is a suitable and more effective vaccine candidate than bPIV3 for protecting humans, especially infants, from the disease-causing agent hPIV3. As hPIV3 infects infants as young as 2 months of age, a vaccine for this disease-causing agent has to be administered prior to the first exposure of the infants to hPIV3. However, infants younger than 2 months possess passively acquired maternal antibodies that may interfere with the "take" of the vaccine and result in a lowered immune response. Thus, high immunogenicity and effectiveness and low disease-causing ability of hPIV3 vaccines are crucial to their success in infants. The currently tested bPIV3 vaccine may thus be enhanced antigenically by introducing the hPIV3 surface glycoproteins responsible for eliciting the neutralizing antibody response, while maintaining the attenuation phenotype resulting in the absence of disease.

Future experiments will also aim at collecting further evidence to support the use of bPIV3 as a RNA virus vector to express and deliver foreign antigens for disease prevention in humans. The specific nucleotide changes in the L gene of r-bPIV3 could be utilized to introduce further attenuation into bPIV3-vectored vaccines. In particular, r-bPIV3 will be evaluated for use as a bi- or multivalent virus vaccine vector expressing the surface glycoproteins of two or more human PIVs.

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